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Earthworm invasion in North America: food resource competition affects native millipede survival and invasive earthworm reproduction.

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Abstract

The invasive non-native earthworm *Amynthas agrestis* (Goto and Hatai 1899) has recently been documented invading forests of the Appalachian Mountains in the southeastern United States. This epigeic earthworm decreases the depth of organic soil horizons, and this may play a role in the decrease of millipede richness and abundance associated with *A. agrestis* invasion. To investigate the mechanisms behind these effects, *A. agrestis* and the millipede *Sigmoria ainsliei* (Xystodesmidae) were placed into microcosms with soil and either L horizon, F and H horizon, or a combination L/FH treatment. Microcosms were destructively sampled and reconstructed with the same treatments every four weeks to assess faunal fresh weight change and survival. Soils from earthworm treatments were wet-sieved for cocoons to assess treatment effects on reproduction. On average, millipede mortality occurred 88 days sooner in treatments that did not have FH horizon material, and within all litter treatments millipedes tended to survive longer when *A. agrestis* was absent. Earthworms maintained higher fresh weight in L/FH than FH or L treatments. With a single exception, no *A. agrestis* cocoons were recovered from microcosms that also contained *S. ainsliei*. The results suggest that *A. agrestis* and *S. ainsliei* may compete for food resources, particularly the smaller particle material in the FH horizons of the forest floor. Millipedes may exert some biotic resistance to *A. agrestis* invasion, as diminished earthworm fecundity was observed in experimental units containing both species.

Keywords: Millipede; earthworm; *Sigmoria; Amynthas;* competition; food preference; invasive species

1 Introduction

Non-native earthworm invasion is a truly global phenomenon in which invasive earthworm species are invading every continent, except Antarctica (Hendrix et al., 2008). These earthworm species also have origins on every continent, except Antarctica. In North America, earthworms of Asian origin (the genera *Amynthas*, *Metaphire*, *Pheretima*, and *Pithemera*) have recently been documented in the northeastern (Steinberg et al., 1997; Burtelow et al., 1998; Bohlen et al., 2004a,b), central (Snyder, unpublished results), and southeastern (Callaham et al., 2003; Snyder et al., 2011) regions of the United States, although these earthworms have been known in North America since the early 20th century (Garman, 1888; Gates, 1937).

Earthworm invasion can significantly alter forest ecosystems. Physical changes to the forest floor through consumption of organic horizons, mixing of organic and mineral horizons, and burrowing and casting activities can impact biogeochemical cycling (Bohlen et al., 2004a,b,c). Earthworm invasion can also impact soil fauna communities through competition and through the significant alteration of soil profile and structure (Bohlen et al., 2004b,c; Frelich et al., 2006). Although much is known about the interactions of invasive earthworms with soil micro- and mesofauna, less is known about interactions with detritivorous macrofauna, such as millipedes (Migge-Kleian et al., 2006). Bonkowski et al. (1998) found that earthworms benefited from consuming millipede (*Glomeris marginata*) fecal pellets in a European Beech forest. However, in a microcosm experiment, millipedes were negatively affected by earthworms (*A. corticis*), but earthworms may have similarly consumed millipede fecal material (Snyder et al.,

2009). Snyder et al. (2009) found that although the millipede *Pseudopolydesmus erasus* was epigeic and *A. corticis* was endogeic, *P. erasus* acquired less C during the four-week course of the experiment in the presence of *A. corticis*, and it is likely that over longer time scales, this C deficit could affect growth, weight maintenance, survival, and/or reproductive output.

Amynthas gracilis invasion in forests of New York, USA was found to reduce O horizon organic matter (Steinberg et al., 1997; Burtelow et al., 1998). Similarly, invasion of *A. agrestis* in the Great Smoky Mountains, USA, reduced the depth of the FH horizon (a combination of the F and H horizons) (Snyder et al., 2011). Millipedes reside in and consume FH horizon, and Snyder et al. (2011) found that millipedes were negatively affected by this *A. agrestis* invasion, both in terms of abundance and species richness. The field observations of Snyder et al. (2011) motivated us to explore the mechanisms behind this interaction, and a microcosm experiment was performed to test whether these two taxa competed for food resources in the L or FH horizons, and whether earthworms and millipedes benefited from the presence of these resources. This microcosm experiment was novel in its approach towards creating a longer-term study (i.e., Months instead of weeks). Earthworms, and to a lesser extent millipedes, burrow in the soil and this prevents regular monitoring of faunal survival and fresh weight without causing disturbance. In order to facilitate data collection, all microcosms were destructively sampled every four weeks and fauna were put into newly constructed microcosms of the same treatment.

2 Methods

91 Millipedes and earthworms were collected by manually searching through leaf
92 litter at the Great Smoky Mountains Institute at Tremont (Blount Co., Tennessee, USA;
93 35°38'22" N, 83°41'17" W), within the Great Smoky Mountains National Park (GSMNP)
94 in early June 2007. Earthworms and millipedes were kept separate during transport to the
95 laboratory. The two taxa were also stored separately until the beginning of the
96 experiment in containers with soil and litter from the collection site.
97 Microcosms consisted of 1 l transparent plastic containers with perforated snap-on
98 lids. Each microcosm received 500 ± 5 g of air dried soil that was then mixed with 70 ±
99 5 mL tap water. Soil was a commercially acquired ultisol (USDA soil taxonomy) from
100 the top 25 cm of a recently cleared forested site in Clarke Co., GA, USA. Soil was
101 screened through a 4.75 mm sieve to remove large aggregates and rocks. Litter was
102 previously collected from GSMNP and defaunated via Berlese extraction for 72 hours,
103 followed by air-drying. Dominant tree species at the litter collection site were *Acer* spp.,
104 *Quercus* spp., *Liquidambar styraciflua*, *Liriodendron tulipifera*, and *Pinus strobus*
105 (Snyder et al., 2011).
106 Litter treatments were defined by particle size: litter was 4.75 mm sieved to
107 separate unfragmented leaves (L horizon) from fragmented and partially decomposed
108 organic matter (FH, combined F and H horizons). Large rocks, twigs, seeds and nuts
109 were discarded. Organic layer treatments were L (15 ± 0.1 g of L horizon), L/FH (7.5 ±
110 0.1 g each of L and FH horizon), or FH (15 ± 0.1 g of FH horizon). Litter was misted
111 with a standard quantity (~7 mL) of tap water when microcosms were constructed. Three
112 fauna treatments were established: two *Amynthas agrestis* individuals (mean fresh weight
113 0.86 ± 0.036 g each); one adult male *Sigmoria ainsliei* (mean fresh weight 2.26 ± 0.038

114 g); and two *A. agrestis* and one *S. ainsliei* together. *Amynthas agrestis* were all clitellate
115 or pre-clitellate. All individuals were approximately the same size and due to the annual
116 nature of their life cycle (Reynolds, 1978; Callaham et al., 2003; Snyder et al., 2011) all
117 individuals were similar in age. Individuals were randomly assigned to treatments with 6-
118 7 replicates for a total of 76 microcosms. However, at the end of the experiment four
119 experimental units were found to contain *Amynthas corticis* rather than *A. agrestis*; these
120 were excluded from subsequent analyses.

121 All microcosms were kept in the dark at 20˚C (± 2ºC). Each microcosm was
122 misted with tap water weekly, except early in the incubation when microcosms were
123 misted every 3 d. Incubation began in June 2007 and continued until all fauna died
124 (except *A. corticis* mentioned above).

125 Microcosms were destructively sampled every four weeks. After destructive
126 sampling, new microcosms were constructed and the surviving fauna were weighed and
127 placed into the new microcosms. Earthworms were rinsed in tap water to remove soil
128 and gently dried on a paper towel prior to weighing. If any fauna (earthworm or
129 millipede) from the original treatment were alive, then a new microcosm was constructed,
130 if all fauna in a particular microcosm had died, then that microcosm was terminated. In
131 this way, longevity of every individual could be assessed. Soils from treatments that
132 included earthworms were wet-sieved through a 2 mm sieve to assess cocoon production.
133 After the first cocoons collected were found to be only slightly larger than 2 mm in
134 diameter, a 1.4 mm sieve was employed to ensure cocoon capture.

135 Millipede and earthworm survival and fresh weight data were analyzed using a
136 general linear model (GLM), with the LSMEANS option for post-hoc tests. Data used in

137 the GLM analysis for earthworm survival were the calculated average days of survival
138 for the two worms in each microcosm. Fresh weight changes through 12 weeks
139 (millipedes) and 16 weeks (earthworms) were analyzed using a repeated measures
140 analysis; beyond this point there were insufficient replicates for robust analyses. Cocoon
141 production was assessed with a t-test comparing between Months 1-3 and 4-7, and GLM
142 comparing between Months 4, 5, 6, and 7. All statistical analyses were completed in SAS
143 (Version 9.2).
144
145 **3 Results**
146 *3.1 Survival and Growth*
147 Millipedes lived a mean time of 136.8 ± 10.6 d (n = 36) from the beginning of the
148 experiment (Fig. 1). The overall model testing fauna and litter effects was significant (P
149 = 0.0002). Millipede survival was significantly affected by litter ($P < 0.0001$), with
150 survival time significantly decreased in L relative to FH ($P < 0.0001$) and L/FH (P =
151 0.0012). However, differences in millipede survival times between L/FH and FH
152 treatments were not statistically significant (P = 0.0567). There was a trend for
153 earthworm presence to decrease millipede survival time, but this was not statistically
154 significant (P = 0.0750). Specifically, when *A. agrestis* was absent, millipedes survived
155 an average of 26 days longer in L/FH and 54 days longer in FH (Fig. 1). Overall,
156 millipedes survived 47.4% (69 days) longer in the L/FH treatment and 58.1% (106 days)
157 longer in the FH treatment relative to the L treatment. There was no interaction between
158 litter and fauna (P = 0.4655).

159 Mean time to *A. agrestis* mortality was 117.9 ± 4.1 d (n = 36 experimental units)
160 from the initiation of the experiment. The first and second *A. agrestis* mortality within
161 each experimental unit were 31.8 ± 5.6 d apart (n = 36). There was no evidence for
162 earthworm survival being affected by millipede presence or litter type (P = 0.2771, Fig.
163 2). In microcosms with both earthworms and millipedes, at least one earthworm survived
164 longer than the millipede in every replicate.
165 Millipede fresh weight (Fig. 3A) did not differ between treatments at the
166 beginning of the experiment (P = 0.5294) or at the last measurement before mortality (P
167 = 0.9010). There were significant differences between litter treatments but earthworms
168 did not impact millipede fresh weight (Fig. 3A, Table 1, analyzed through week 12).
169 Millipede fresh weight increased significantly more in FH relative to L treatments (P =
170 0.0100), but neither were significantly different from L/FH treatments (FH vs. L/FH P =
171 0.1351; L vs. L/FH P = 0.1412). Within-subjects tests for effects over time and
172 interactions with time were all non-significant (data not shown).
173 Earthworm fresh weight (Fig. 3B) did not differ between treatments at the
174 beginning of the experiment (P = 0.6190). A significant impact of litter (P = 0.0180), but
175 not of millipede treatments (P = 0.9531, Table 1), was observed in fresh weight changes
176 through week 16: *A. agrestis* maintained a higher fresh weight in L/FH than in FH and L
177 treatments. Earthworm fresh weight decreased over time (Table 2, Fig. 3B, $P < 0.0001$),
178 anda time by litter interaction was also significant (P = 0.0211).
179 *3.2 Earthworm cocoon production*
180 Cocoons were detected beginning in the fourth month and in every subsequent
181 month of incubation (Fig. 4). In Months 4-7, microcosms in which cocoons were

182 recovered contained 2.06 ± 0.44 cocoons; this was a significant increase over the zero
183 cocoons recovered during Months 1-3 (t-test, n = 17, $P = 0.0003$). Numbers of cocoons
184 recovered in Months 4-7 were not significantly different from one another (GLM, $P =$
185 0.8952). Cocoons were recovered from microcosms that began a month with either one or
186 two earthworms, but the number of cocoons per microcosm was not significantly
187 different due to this factor ($P = 0.5381$). There were a total of 28 cocoons recovered
188 during the experiment, and only one of these was recovered from a microcosm that also
189 contained a live millipede. Three cocoons were recovered in millipede treatments after
190 millipede mortality. Litter treatment did not influence the number of cocoons recovered
191 per microcosm ($P = 0.7868$).

192 **4 Discussion**

193 Based on inferences from field (Snyder et al., 2011) and microcosm (Snyder et
194 al., 2009) studies that Asian invasive earthworms may compete with native North
195 American millipedes, we designed a microcosm experiment to evaluate longer-term
196 interactions between two species focusing on the potential for food competition. 'Longer-
197 term' in this case is relative to most microcosm experiments and also to the putative life-
198 span of these taxa, i.e., the experiment continued for months rather than weeks. This
199 methodology had the advantage of allowing measurement of fresh weight and survival
200 while limiting the frequency of disturbance. Although the disturbance to the microcosms
201 may seem substantial - the entire microcosm was destroyed and replaced – in reality the
202 stress to the organisms was quite brief and limited as much as possible. In practice, each
203 organism was quickly located, weighed, and placed into a new microcosm in a matter of
204 seconds. The alternative of searching for surviving individuals, weighing, and returning

205 to the same container would have the potential of differentially disturbing experimental
206 units depending on how quickly and easily individual organisms were discovered,
207 collected, and measured.

208 Presence of FH material was important for *Sigmoria ainsliei*: millipede survival
209 time decreased greatly without FH material and biomass increased the most in the FH
210 treatment. However, earthworm fresh weight, but not survival, was highest in the
211 treatment with both particle sizes (L/FH). In FH and L/FH, we observed that *A. agrestis*
212 consumed nearly all FH material within each four-week time period. This finding is
213 consistent with field observations and data showing that a decrease in FH horizon
214 correlates with *A. agrestis* invasion (Snyder et al., 2011), and supports the hypothesis that
215 *A. agrestis* directly causes this decrease through consumption.

216 In L/FH and FH treatments, there was also a trend that *S. ainsliei* survived a
217 shorter amount of time when *A. agrestis* was present, but this was not statistically
218 significant. However, we propose that from biological standpoint, this may indeed be a
219 relationship worthy of further exploration. Interestingly, when both species were present,
220 millipedes almost always died first, and this suggests that when the two are in close
221 proximity, the invasive earthworms may outcompete millipedes and eventually exclude
222 them. In these same litter treatments (FH and L/FH) there was also a very weak trend that
223 *A. agrestis* survived longer in treatments without millipedes. However, in L treatments,
224 *A. agrestis* tended to survive longer in the presence of millipedes, suggesting that
225 earthworms may benefit from millipede presence in L treatments, probably through
226 consumption of litter that had been processed by millipedes, as has been observed in
227 other studies (Bonkowski et al., 1998; Snyder et al., 2009). Earthworms were also

228 observed to burrow into mineral soil during the incubations, and may have been able to
229 exploit organic matter in the mineral soil in addition to resources supplied on the soil
230 surface (Zhang et al., 2008; Callaham et al., unpublished results). This behavior is
231 consistent with the findings of Zhang et al. (2008) who showed that *Amynthas* had
232 substantial dietary flexibility, and this possible additional source of organic matter may
233 help explain why earthworm survival was not affected by litter treatment, as well as why
234 the effect of millipede presence was not statistically significant. When availability of
235 aboveground resources was limited, *A. agrestis* may have burrowed and consumed soil
236 organic matter, while adult *S. ainsliei* were restricted to feeding on surface organic
237 horizons. Millipedes appear to have inhibited reproductive potential, possibly through
238 this same mechanism. In the presence of millipedes, earthworms may have spent more
239 energy burrowing to access lower quality food resources. This combination may have led
240 to less energy being available to devote to cocoon production.

241 *4.1 Conclusions and future perspectives*

242 Overall, the data from the present study are consistent with, and help to elucidate,
243 observations from field studies (Snyder et al., 2011), and other lab experiments with
244 invasive earthworms and millipedes (Snyder et al., 2009). *Amynthas agrestis* invasions
245 being associated with decreased F/H horizon depth and decreased millipede abundance
246 (Snyder et al., 2011) served as a starting point to ask questions about what the potential
247 mechanisms behind these relationships might be. From our microcosms, we now have
248 evidence that two of the organisms involved in the field study will consume the same
249 food sources, and that when they are kept in proximity to one another, these organisms
250 affect one another's longevity and reproductive output. Although microcosms are, of

251 necessity, quite simple relative to the natural systems they are meant to simulate, they can
252 nevertheless offer important insights particularly into mechanistic relationships (Drake
253 and Kramer, 2012; Cadotte et al., 2005). We suggest that our study has uncovered just
254 such a mechanistic relationship between *A. agrestis* and *S. ainsliei*, but we also
255 recommend that much more detailed work should be undertaken to examine the trophic
256 ecology and resource use of these organisms in their native habitats. Such work will be
257 crucial if we are to have fuller understanding of effects of earthworm invasion, and
258 imperative to the future development of successful management approaches to control
259 earthworm invasions in the Southern Appalachian Mountains.

260 Our data lends support to the hypothesis that earthworms and millipedes compete
261 for partially decomposed leaf material, but many questions remain. Greater cocoon
262 production in the absence of millipedes supports the competition hypothesis and suggests
263 that millipedes may provide some biotic resistance to invasion. Future studies could
264 offset the natural variability in earthworm and millipede mortality, and improve their
265 statistical power, by including more replicates. Additionally, initiating treatment
266 conditions on younger individuals may produce stronger responses. Maintenance of
267 laboratory cultures (Lowe and Butt, 2005) will be a critical step in our ability to perform
268 more of these experiments.

269

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348

Figure Captions

Fig. 1. Mean survival (± SE) of *Sigmoria ainsliei* from initiation of the incubation with (M+W) and without (M) earthworms. Litter treatments were litter (L), litter and FH material (L/FH), and FH only (FH).

Fig. 2. Mean survival (± SE) of *Amynthas agrestis* from initiation of the incubation with (W+M) and without (W) millipedes. Litter treatments were litter (L), litter and FH material (L/FH), and FH only (FH).

Fig. 3. Mean fresh weight change of surviving fauna (± SE) since the beginning of the experiment, expressed as percent of initial mass: millipedes (A) and earthworms (B) in different litter treatments through 12 weeks (millipedes) or 16 weeks (earthworms) of the incubation. Different letters indicates significant *P*-values at α=0.05 within one sampling time.

Fig. 4. Cocoon production by *Amynthas agrestis* over the duration of the incubation. Mean (± SE) number of cocoons recovered per microcosm (A) and number of earthworm-containing microcosms from which cocoons were (shaded) and were not (open) recovered (B). Cocoon recovery in Months 4-7 was greater than 0 (Months 1-3, $P = 0.0003$; $n = 17$).

372 Table 1. Results of repeated measures GLM analyses tests of hypotheses for between
373 subjects effects on fresh weight change from initiation of the incubation. Fresh weight
374 was measured every four weeks. Millipede data were analyzed until week 12 and
375 earthworm data until week 16. Significant *P*-values at α=0.05 indicated by an asterisk.
376

Source	df	SS	MS	*F*	*P*
Millipede Fresh Weight					
Fauna treatment	1	3.72×10^{-6}	3.72×10^{-6}	0.00	0.9912
Litter treatment	2	0.21553	0.10777	3.59	0.0438*
Fauna * Litter	2	0.03781	0.01891	0.63	0.5411
Error	23	0.68954	0.02997		
Earthworm Fresh Weight					
Fauna treatment	1	0.00274	0.00274	0.00	0.9531
Litter treatment	2	7.73328	3.8666	5.00	0.0180*
Fauna * Litter	2	1.19285	0.59642	0.77	0.4761
Error	23	14.6819	0.77273		

377
378

Table 2. Results of repeated measures GLM analyses tests of hypotheses for within subjects effects on earthworm fresh weight change from initiation of the incubation. Fresh weight was measured every four weeks and analyzed until week 16. Significant *P*-values at $\alpha=0.05$ indicated by an asterisk.

Source	df	SS	MS	*F*	*P*
Time	3	19.2486	6.41619	86.54	< 0.0001*
Time * Fauna	3	0.18038	0.06013	0.81	0.4930
Time * Litter	6	1.21529	0.20255	2.73	0.0211*
Time * Fauna * Litter	6	0.07883	0.01314	0.18	0.9820
Error (Time)	57	4.22590	0.07414		